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Journal of Controlled Release 65 (2000) 93–103

journal of controlled release

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Inner core segment design for drug delivery control of thermo-responsive polymeric micelles

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Received 22 July 1999; accepted 18 September 1999

Abstract

Modification of the thermo-responsive behavior of polymeric micelles for specific drug delivery functions was investigated using combinations of micellar inner cores and outer shell polymer chemistries. Polymeric micelles comprised of AB block copolymers of PIPAAm (poly(*N*-isopropylacrylamide)) with either PBMA (poly(butyl methacrylate)) or PSt (polystyrene) were employed. PIPAAm–PBMA and PIPAAm–PSt block copolymers formed a core-shell micellar structure after dialysis of the block copolymer solutions in organic solvents against water at 20°C. The hydrophobic drug, adriamycin, (ADR) was loaded into the inner core of the polymeric micelles by dialysis. The polymers showed reversible intermicellar dispersion/aggregation in response to temperature cycles through an outer polymer shell lower critical solution temperature (LCST for PIPAAm=32.5°C), observed by DLS (dynamic light scattering) and transmittance measurements. Upon heating above the LCST, PIPAAm–PBMA micelles exhibited an abrupt increase in micropolarity and an abrupt decrease in microrigidity sensed by pyrene and 1,3-*bis*(1-pyrenyl)propane (PC_3P), respectively. In contrast, PIPAAm–PSt micelles maintained constant values with lower micropolarity and higher microrigidity than those of PIPAAm–PBMA micelles over the temperature range 20 to 40°C. From these results, structural deformations produced by outer shell polymer structural change with temperature cycles through the LCST are proposed for the PBMA core possessing a lower T_g (ca. 20°C) than the outer shell PIPAAm LCST. The PSt core with a much higher T_g (ca. 100°C) than the outer shell LCST retained its structure, regardless of outer shell changes. PIPAAm–PBMA micelles released ADR only when heated above the LCST, while PIPAAm–PSt micelles did not. Cell cultures treated with PIPAAm–PBMA micelles loaded with ADR showed high in vitro cytotoxicity when heated above the LCST, while PIPAAm–PSt micelles loaded with ADR expressed very low in vitro cytotoxicity irrespective of temperature change through the LCST. The nature of hydrophobic segments comprising the micelle inner core offers an important control point for thermo-responsive drug release and the drug activity of the thermo-responsive polymeric micelle.

Keywords: Poly(*N*-isopropylacrylamide); Poly(butyl methacrylate); Polystyrene; Polymeric micelle; Block copolymer; Thermo-response; Drug delivery; Adriamycin; In vitro cytotoxicity

1. Introduction

It is well known that poly(*N*-isopropylacrylamide) (PIPAAm) in aqueous solution exhibits a reversible thermo-responsive phase transition at 32°C [1],

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0168-3659/00/$ – see front matter
PII: S0168-3659(99)00242-4

termed the lower critical solution temperature (LCST). PIPAAm is water-soluble and hydrophilic, exhibiting an extended chain conformation below its LCST, yet undergoing a phase transition to an insoluble and hydrophobic aggregate above the LCST. This phase transition occurs within a narrow temperature range and is reversible. Utilizing this thermo-responsive property, PIPAAm and its gels have been studied for on–off drug release [2–4], chromatography systems [5–7], and attachment/detachment of cultured cells [8–10].

AB-type block copolymers consisting of a PIPAAm segment and a hydrophobic segment can form core-shell micellar structures below the PIPAAm LCST. This polymeric micellar structure comprises a hydrophilic outer shell of hydrated PIPAAm segments and a hydrophobic inner core. The inner core can be loaded with hydrophobic drugs, while the PIPAAm outer shell plays the role of aqueous solubilization and temperature-responsiveness. Polymeric micelle size ranges are tailored by block copolymerization. Careful selection of block segments and their chain lengths can produce micelles that inhibit non-selective scavenging by the reticuloendothelial system (RES) and can be utilized as tumor-targetable drug carriers based on the EPR (enhancement of permeability and retention) effect [11,12]. We have already shown that an anticancer drug, adriamycin (ADR) in polymeric micelles formed from block copolymers of poly(ethylene oxide) (PEG) and poly(*l*-aspartate) selectively accumulates at solid tumor sites by a passive targeting mechanism utilizing the EPR effect and avoidance of the RES. This property is attributed to the hydrophilic outer PEG chains and favorable micellar size (<100 nm) [13,14].

Polymeric micelles with a hydrophilic PIPAAm outer shell and a favorable size (<100 nm) may exhibit specific targeting of solid tumor sites by a similar passive targeting mechanism. Furthermore, the thermo-responsiveness of these micelles can increase the targeting efficiency via a stimuli-responsive targeting process that utilizes local heating at solid tumor sites. Thermo-response is expected to exhibit multiple targeting functions: both a passive and a stimuli-responsive targeting mechanism, plus the therapeutic effect of hyperthermia by local heating. Moreover, hyperthermia has been reported to enhance the cytotoxicity of some anticancer drugs by synergistic effects in vitro and in vivo [15,16]. Weinstein and coworkers utilized thermo-sensitive liposomes to achieve temperature modulated, targeted drug delivery [17]. However, conventional liposome formulations have only limited value in vivo due to non-selective scavenging by the RES and slow responsiveness to the temperature changes [17,18]. The strategy using thermo-responsive polymeric micelles can achieve temporal drug delivery control: the drug is released and expresses its bioactivity only for a time period defined by local heating and cooling.

In order to design and facilitate a reversibly temperature-responsive micelle for drug delivery, we have exploited polymer micelle formation, structural stability and temperature-responsiveness associated with chemical structures of PIPAAm copolymers [19–24]. Also, we have reported the possibility of thermo-responsive in vitro drug release and cytotoxicity using PIPAAm–PBMA (poly(*N*-isopropylacrylamide)–*b*-poly(butyl methacrylate)) polymeric micelles loaded with ADR [23].

We report and discuss herein the ON/OFF control of drug delivery associated with thermo-responsive micellar structural changes, focusing on the combined properties of the outer shells and the inner cores. In particular, we propose that this combination correlates closely with the drug release behavior of the thermo-responsive polymeric micelles. Physical and chemical control of the inner core, such as design of polymer flexibility, hydrophobicity and degradability will regulate the inner core behavior affected by outer shell change upon temperature cycling. Exploiting the relationships between the outer shell–inner core combination and drug action of the polymeric micelles will offer important new information to design improved thermo-responsive polymeric micelles for fine control of drug delivery as well as deepen the understanding of drug action mechanisms through polymeric micelle carriers.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (IPAAm), kindly provided

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2.2. Synthesis of
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by Kohjin, Tokyo, Japan, was purified by recrystallization in hexane and dried in vacuo at room temperature. 2-Mercaptoethanol (ME), styrene (St) and tetrahydrofuran (THF) were obtained from Wako Pure Chemicals (Tokyo, Japan) and purified by the standard methods. Butyl methacrylate (Tokyo Kasei Co. Ltd.) and 3-mercaptopropionic acid (MPA, Aldrich, Milwaukee, WI, USA) were distilled under reduced pressure. *N*-Ethylacetamide was purchased from Tokyo Kasei. Benzoylperoxide (BPO) and triethylamine (TEA) were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). Pyrene and 1,3-*bis*(1-pyrenyl) propane (PC_3P) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Synthesis of AB block copolymers of PIPAAm with poly(butyl methacrylate) (PBMA) or polystyrene (PSt)

Hydroxyl semitelechelic PIPAAm (PIPAAm–OH) was synthesized by telomerization using ME as a chain transfer agent [2,25–28]. IPAAm (300 mmol), ME (6 mmol) and BPO (0.504 mmol) were dissolved in THF (100 ml) and repeatedly degassed under reduced pressure in freeze–thaw cycles. Polymerization was carried out at 70°C for 4 h, and then was stopped by freezing. After evaporation of most of the THF, polymers were obtained by precipitation into an excess of diethyl ether and dried in vacuo. The dried polymer was dissolved in water and fractionated by membrane ultrafiltration (MWCO= 20,000 and 10,000, ultrafiltration cell UHP-76K, ultrafilter, ADVANTEC) at 4°C. Semitelechilic PIPAAm molecular weight was determined by gel permeation chromatography (GPC, TOSOH, SC-8020, polystyrene standard) in DMF containing LiBr (10 mM) (elution rate: 1 ml/min) at 40°C.

Both carboxyl terminated PSt (PSt–COOH) and PBMA (PBMA–COOH) were prepared by telomerization using MPA as a chain transfer agent [21]. PBMA–COOH was synthesized and purified as previously described [23]. Styrene (1.92×10^{-1} mol), MPA (1.54×10^{-3} mol) and *N,N′*-azo-*bis*-isobutyronitrile (AIBN, 5.50×10^{-5} mol) were dissolved in DMF (40 ml). This solution was repeatedly degassed under reduced pressure in freeze–thaw cycles. Polymerization was carried out at 65°C for 20 h and stopped by freezing. After evaporation of most of the DMF, polymers were recovered by precipitation into an excess of methanol and dried in vacuo. PSt–COOH molecular weight was determined by gel permeation chromatography (GPC, TOSOH, SC-8020, polystyrene standard) in DMF containing LiBr (10 mM) (elution rate: 1 ml/min) at 40°C.

A block copolymer of PIPAAm and PSt (PIPAAm–PSt) was obtained by a condensation reaction between the terminal carboxylic end group of semitelechilic PSt–COOH (MW 2,400) and the hydroxyl group of semitelechilic PIPAAm–OH (MW 10,000) [21]. Block copolymers of PIPAAm–PBMA were obtained by reactions of hydroxyl groups of this same PIPAAm with activated terminal groups of PBMA (MW 8,900) [23]. Resulting products were precipitated twice in a large excess of diethyl ether and then precipitated again in warm water (30°C) in order to obtain pure block copolymers of PIPAAm–PBMA. The molecular weight of PIPAAm–PBMA (MW 19,400) was determined by gel permeation chromatography (GPC, TOSOH, SC-8020, polystyrene standards) in DMF containing LiCl (10 mM) (elution rate: 1 ml/min) at 40°C.

2.3. Micelle formation by PIPAAm block copolymers

Solutions of PIPAAm–PSt and PIPAAm–PBMA were prepared by dissolving each copolymer (19 mg) in *N,N*-dimethylacetamide (DMAc, 3 ml) and *N*-ethylacetamide (3 ml), respectively. These solutions were placed in dialysis bags (regenerated cellulose, MWCO=12,000~14,000) and dialyzed against distilled water at 20°C for 24 h.

2.4. Optical transmittance measurements

Optical transmittance of aqueous polymer solutions (5,000 mg/l) at various temperatures was measured at 542 nm with a UV spectrometer (Ubest-30, Japan Spectroscopic Co. Ltd., Tokyo, Japan). Sample cells were thermostated with a circular water jacket from 10 to 40°C. LCSTs of polymer solutions were defined as the temperature producing a 50% decrease in optical transmittance.

2.5. Fluorescence measurements

Fluorescence spectra were recorded using a spectrofluorometer (FP-770, Japan Spectroscopic Co., Ltd., Tokyo, Japan). The temperature of a water-jacketed cell holder was controlled with a thermostated circulating bath. Pyrene and PC_3P were used as hydrophobic fluorescent probes [19,29–31]. Pyrene solution in acetone (4.8×10^{-4} M, 5 μl) or PC_3P solution in acetone (1.3×10^{-4} M, 5 μl) was added to aqueous polymer solutions (20,000 mg/l, 4 and 3 ml, respectively). These samples containing pyrene (ca. 6×10^{-7} M) or PC_3P (ca. 2.2×10^{-7} M) were kept for 24 h at 20°C before measurements. Fluorescence excitation was carried out at 333 nm (PC_3P) and 340 nm (pyrene). Emission spectra were recorded over 350 to 600 nm. Excitation and emission band widths were 10 and 3 nm, respectively. From pyrene emission spectra, the intensity (peak height) ratios (I_1/I_3) of the first band (374 nm) to the third band (385 nm) were analyzed as a function of concentration of the polymer solutions and temperature (heating or cooling rate = 1°C/min). The PC_3P excimer emission to monomer emission ratios (I_E/I_M) were calculated from excimer intensity (I_E) at 474 nm and monomer intensity (I_M) at 378 nm [19].

2.6. Adriamycin (ADR) loading

PIPAAm–PBMA or PIPAAm–PSt block copolymer (19 mg) and ADR hydrochloride (19 mg) were dissolved separately in 1.5 ml of *N*-ethylacetamide or DMAc, respectively. Triethylamine (6.0 μl) was added dropwise to the ADR solution, and this ADR solution was then added to the block copolymer solution. The mixed solution was dialyzed against water at 20°C for 48 h. The resulting red solution was ultrafiltered three times and the resulting absorbance of loaded and unloaded ADR was measured at 500 and 485 nm, respectively [23]. Drug loading efficiency was calculated by the weight ratio of ADR in micelles to micelles loaded with ADR.

2.7. In vitro drug release

ADR released from micelles was isolated from micellar media using an ultrafiltration membrane (MWCO = 100,000, ultrafiltration cell, Millipore) and measured in aqueous solutions below and above the micelle LCST using absorbance at 485 nm in a time-course procedure.

2.8. In vitro cytotoxicity measurements of micelles loaded with ADR

In vitro cytotoxic activity of free ADR, blank micelles or the micelles loaded with ADR was measured using cultured bovine aorta endothelial cells. Bovine aorta endothelial cells were obtained by a previously reported method using dispase for cell dissociation from freshly harvested bovine aorta [9]. The primary cultures were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone, and incubated at 37°C in a humidified atmosphere with 5% CO_2. Fungizone was discontinued on the seventh day of culture. Cells were routinely split at a ratio 1:4 and carried in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin [32]. Cells were plated at a density of 3×10^4 cells/well and were then exposed with free ADR, blank micelles or micelles loaded with ADR below and above the LCST for 4 days. In order to assay cytotoxicity of free ADR, blank micelles or micelles loaded with ADR, culture media was replaced with 10% FBS-supplemented phenol red-free DMEM containing 10% alamar Blue, a dye which when subjected to reduction by cytochrome c activity changes color from blue to red [33]. After an incubation, reduction of the dye was measured by optical absorbance at 560 and 600 nm.

3. Results and discussion

3.1. Core-shell micellar structure

Our research group [19,20,25,28] and Hoffman and coworkers [34–36] have succeeded in demonstrating LCST solution control of PIPAAm random copolymers by incorporating hydrophilic or hydrophobic comonomers. Hydrophilic groups increase the copolymer LCST values and slow down phase transition kinetics by stabilizing polymer dissolution [34,35]. By co
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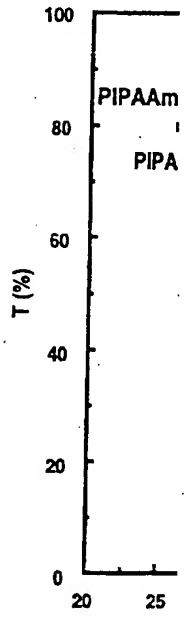

Fig. 1. LCST profil
PIPAAm–PSt micellar
542 nm, [polymer] = 5

[34,35]. By contrast, hydrophobic groups reduce phase transition temperatures [19,28,36]. We have previously reported that the hydrophilic or hydrophobic contribution to the PIPAAm LCST transition was particularly high when such groups were located at one end of the PIPAAm chain [19,20]. The obtained PIPAAm–OH homopolymer exhibited a higher LCST of 34.5°C than unmodified PIPAAm (LCST=32.5°C). However, micelles formed by block copolymers of PIPAAm–PBMA or PIPAAm–PSt showed the same LCST as that for the intact PIPAAm, irrespective of hydrophobic segment incorporation (Fig. 1). This confirms that the block copolymers formed core-shell micellar structures with completely separated phases [19,20]. We have previously reported that micellar structures formed from AB block copolymers comprising the PIPAAm segment with hydrophobic segments show a similar LCST and phase transition kinetics as freely mobile, linear PIPAAm chains [19–21,23]. This is because strong interaction of the hydrophobic segments formed clearly phase-separated micellar structures

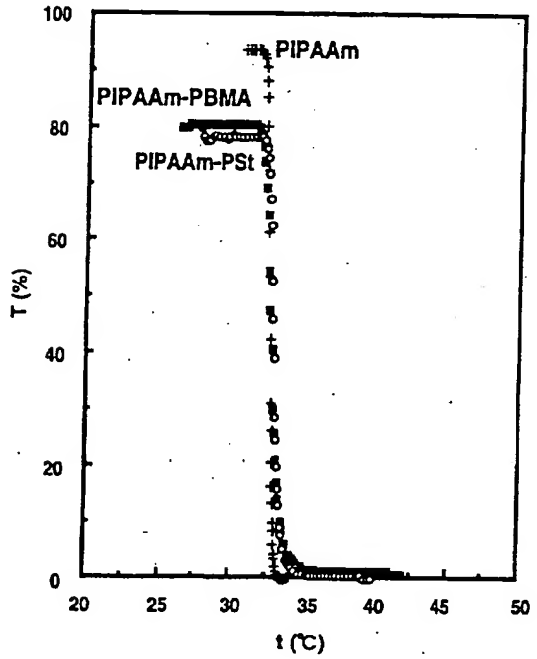

Fig. 1. LCST profiles for PIPAAm, PIPAAm–PBMA and PIPAAm–PSt micellar solutions determined by transmittance at 542 nm, [polymer]=5000 mg/l.

[19–25]. We have already proved that thermo-responsive aggregation/dispersion of these micelles is mediated by a reversible change in the hydrophobic/hydrophilic property of the PIPAAm outer shell in heating/cooling thermal cycles through the LCST using DLS measurements and transmittance measurements [19–21].

3.2. Thermo-responsive inner core deformation

The changes in the inner core properties of PIPAAm–PBMA and PIPAAm–PSt micelles as a function of temperature through the micellar LCSTs were investigated by fluorescence spectroscopy using pyrene and PC_3P as fluorescence probes. The fluorescence spectrum of pyrene at low concentration possesses a vibrational band structure with a strong sensitivity to the polarity of the pyrene environment [37]. The ratio (I_1/I_3) of intensity of the first band (I_1) to that of the third band (I_3) was monitored as a function of temperature above the critical micelle concentration (cmc) [38]. The larger ratio indicates the more polar microenvironment around the pyrene probe. Fig. 2 shows micropolarity changes sensed by pyrene molecules in micelle solutions of PIPAAm–PBMA and PIPAAm–PSt as a function of temperature. PIPAAm solutions demonstrate an abrupt decrease in polarity when the temperature was raised through the LCST, indicating transfer of pyrene into the precipitated polymer-rich phase [19]. By contrast, the micelle solutions of PIPAAm–PBMA showed an increase in polarity above the LCST. Aggregation of collapsed PIPAAm outer shells may induce micelle structural deformation, increasing the pyrene microenvironment polarity, resulting in the observed increase in pyrene polarity above the LCST [19,23]. The polarity change was reversible, responding to a heating/cooling thermal cycle through the LCST. It is thought that the micellar structural deformation producing this change in pyrene partitioning reverted to the initial micelle structure with increasing rehydration of the PIPAAm chains below the LCST [19,23]. On the other hand, micelle solutions of PIPAAm–PSt did not show any change in microenvironment polarity over the tested temperature region. This indicates that glassy inner cores comprising PSt segments do not readily permit a structural change of the inner core upon heating or cooling

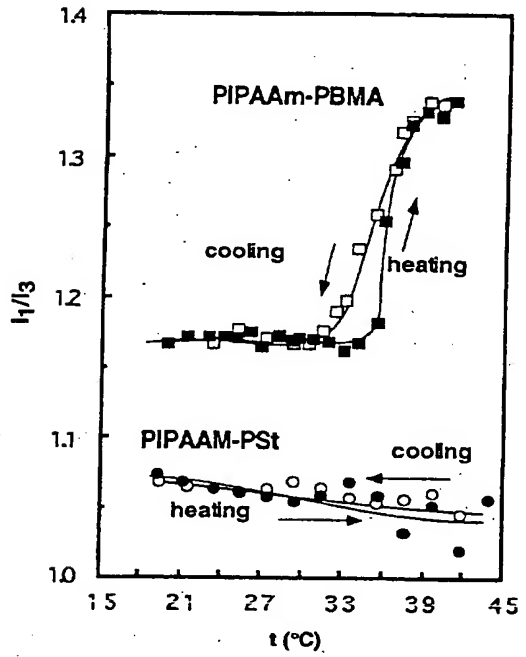

Fig. 2. Plot of the ratio of intensities (I_1/I_3) of the vibrational bands in the pyrene fluorescence spectrum as a function of temperature for PIPAAm–PBMA and PIPAAm–PSt micellar solutions, λ_{ex}=340 nm, [pyrene]=1.6×10^{-7} M, 1°C/min, [polymer]=5000 mg/l.

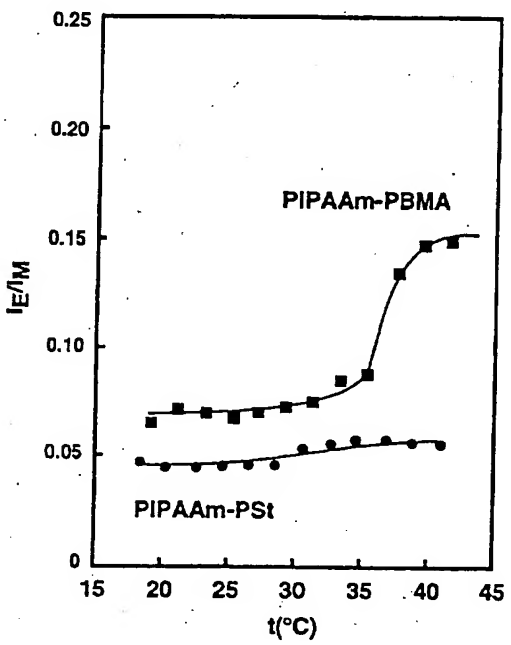

Fig. 3. Plot of the ratio of intensities (I_E/I_M) of the vibrational bands in the PC_3P fluorescence spectrum as a function of temperature for PIPAAm–PBMA and PIPAAm–PSt micellar solutions, λ_{ex}=333 nm, [PC_3P]=2.2×10^{-7} M, 1°C/min, [polymer]=20 000 mg/l.

through the LCST, irrespective of either aggregation or rehydration of PIPAAm outer shells. The PSt micellar core apparently preserves the very stable hydrophobic core structure which is unresponsive to outer shell structural change.

Fig. 3 shows emission spectra of PC_3P in PIPAAm–PBMA and PIPAAm–PSt micelle solutions above their cmc as a function of temperature. The lower I_E/I_M value indicates the more rigid microenvironment around this probe. PIPAAm homopolymer solutions showed a discontinuous decrease in I_E/I_M values as the temperature increased through the LCST, implying a phase transition in PIPAAm chains. This result suggests that the motion of PC_3P is suppressed by the microviscosity created by compact polymer chain aggregation. In contrast, PIPAAm–PBMA micelle solutions exhibited increases in I_E/I_M as the temperature increased through the LCST. This provides evidence for a decrease in inner core rigidity above the LCST due to structural deformation. Furthermore, PIPAAm–PSt micelle solutions demonstrated higher microrigidity than the PIPAAm–PBMA micelle solution over the tested temperature region due to the contribution of more rigid inner cores formed by PSt segments. Moreover, little microrigidity decrease was observed upon heating above the LCST.

3.3. Micellar drug release

Water-insoluble drugs can be physically incorporated and stabilized in the hydrophobic micellar inner core by hydrophobic interaction [21,23,24]. Micelle formation and drug loading resulting from solvent exchange during dialysis are thought to be significantly affected by interaction of the solvents with both polymers and drugs. PIPAAm–PBMA formed polymeric micelles were successfully loaded with ADR (9.6 wt.%) without precipitation by using *N*-ethylacetamide as a good solvent for both the

polymers and AI
[23]. The mixed
ADR in *N,N*-dim
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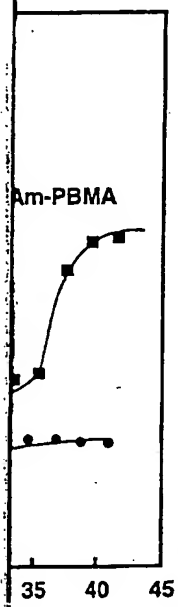

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polymers and ADR, and other selected conditions [23]. The mixed solutions of PIPAAm–PBMA and ADR in *N,N*-dimethylformaldehyde or dimethylsulfoxide precipitated during dialysis against water. In contrast, micelle formation and ADR incorporation (15.2 wt.%) were most successful for PIPAAm–PSt systems with DMAc. PIPAAm–PSt was not soluble in *N*-ethylacetamide. Solutions of PIPAAm–PSt and ADR in *N,N*-dimethylformaldehyde or dimethylsulfoxide also precipitated during dialysis against water.

In order to remove hydrochloride salt groups from ADR, 1.3 molar equivalents of TEA were added to the ADR solution dropwise prior to mixing with a polymer solution. A larger amount of TEA addition resulted in precipitation during dialysis, while addition of less TEA failed to improve ADR loading due to weak hydrophobic interactions.

The micelle–ADR products were obtained as a transparent red solution at ca. 20°C (dialysis temperature). Other higher or lower dialysis temperatures led to precipitates. Intramolecular hydrophilic/hydrophobic PIPAAm block copolymer balance facilitated micelle formation depended also on the solution temperature, because hydrophobic segment aggregation was opposed or assisted by the thermally dependent hydrophilicity and solubility of the PIPAAm segments [19]. The solution temperature (dialysis temperature) of ca. 20°C probably provided a favorable intramolecular hydrophilic/hydrophobic balance for successful micelle formation and drug loading in these conditions.

Fig. 4 shows drug release from PIPAAm–PBMA and PIPAAm–PSt micelles loaded with ADR in response to temperature changes. Drug release from the PIPAAm–PBMA micelle was initiated by heating above the LCST, corresponding well with inner core structural deformation sensed by pyrene and PC_3P upon heating. In a previous report PIPAAm–PBMA micelles were shown to exhibit a small initial burst (ca. 10%) of ADR below the LCST [23]. This initial burst resulted from release of unstable ADR distributed in the PIPAAm outer shell. The initial burst was completely removed by repeated ultrafiltration. The observation of synchronous PIPAAm–PBMA micelle structural changes and ADR release suggests that the inner core structural change induced by aggregation and collapsed PIPAAm upon heating above the LCST elicits ADR release, while highly hydrated

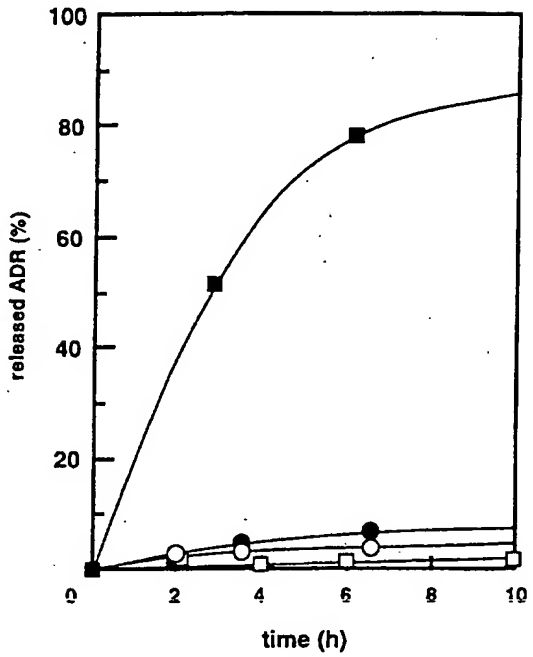

Fig. 4. Drug (ADR) release from PIPAAm–PBMA and PIPAAm–PSt micelles loaded with ADR below and above the LCST, closed and opened squares: PIPAAm–PBMA at 40°C (■) and 4°C (□), respectively, closed and opened circles: PIPAAm–PSt at 40°C (●) and 4°C (○), respectively.

PIPAAm stabilized the ADR load in micellar cores below the LCST. On the other hand, PIPAAm–PSt micelles that preserve their core structures irrespective of outer shell structural change did not show a significant increase in the ADR release rate upon heating above the LCST. Moreover, ADR release from PIPAAm–PBMA micelles switched reversibly between ON and OFF release states in response to temperature changes through the LCST (Fig. 5). PIPAAm–PSt micelles released only a very small amount of ADR on repeated heating and cooling through the LCST (Fig. 5). These results strongly indicate that the inner core structural change in response to PIPAAm outer shell changes is an important determinant for thermo-responsive drug release control.

We have already confirmed that stearoyl-terminated PIPAAm (PIPAAm–C_{18}) micelles released most of the loaded drug relatively fast, even below the LCST, while PIPAAm–C_{18} successfully formed

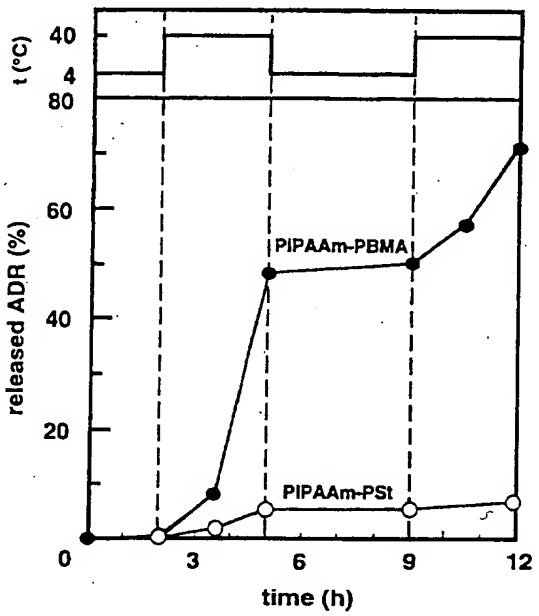

Fig. 5. Drug (ADR) release from PIPAAm–PBMA and PIPAAm–PSt micelles loaded with ADR in response to temperature switching between 4 and 40°C.

core-shell micellar structures loaded with drugs (data not shown). Actually, a ratio of excimer intensity to monomer intensity (I_E/I_M) of PC_3P in the PIPAAm–C_{18} micellar solution showed considerably lower microrigidity ($I_E/I_M=0.127$) compared to the I_E/I_M values (0.067) for either PIPAAm–PBMA micelle solutions or PIPAAm–PSt micelle solutions ($I_E/I_M=0.044$). The alkyl chain ($-C_{18}$) core was not able to retain drugs, even without any heating cycles. In order to reliably utilize the outer shell thermo-response for specific drug delivery, it is important to rationally design the inner core segments. Choosing optimal physical and chemical properties of the inner core segment, such as T_g and hydrophobicity, was found to produce a fine control of drug release from polymeric micelles due to temperature modulation of the core.

3.4. In vitro cytotoxicity

The in vitro cytotoxic activity of PIPAAm–PBMA micelles loaded with ADR (PIPAAm–PBMA/ADR micelles) and PIPAAm–PSt micelles loaded with ADR (PIPAAm–PSt/ADR micelles) is compared in Table 1. We have already reported the possibility of thermally specific drug toxicity of PIPAAm–PBMA/ADR micelles by heating above the LCST [23]. The PIPAAm–PBMA/ADR micelles showed higher cytotoxic activity than that of free ADR above the LCST, while exhibiting lower cytotoxic activity than that of free ADR below the LCST. Blank polymeric micelles of PIPAAm–PBMA and PIPAAm–PSt showed no cytotoxicity, demonstrating that properties of the polymeric micelles themselves did not affect cytotoxicity. The cytotoxic activity of those micelles corresponded well to micelle structural changes and ADR release behavior of each micelle. PIPAAm–PBMA/ADR micelles showed selective cytotoxicity only upon heating above the LCST, whereas PIPAAm–PSt/ADR micelles did not exhibit cytotoxicity, even above the LCST under identical conditions (including incubation time after cell exposure to micelles loaded with ADR (4 days) and ADR concentration (0.1 μg/ml)). These results demonstrate that ADR-loaded polymeric micelles express cytotoxic activity only due to ADR released by core structural changes upon heating above the LCST.

Additionally, the higher cell cytotoxicity of the PIPAAm–PBMA/ADR micelle over that seen for the same amount of free ADR in culture suggests different routes for drug uptake by cells caused by the carrier properties. We have previously reported that hydrophobic PIPAAm chains collapsed above the LCST actively interact with cells, while hydrated PIPAAm chains below the LCST do not [8–10].

Table 1
In vitro cytotoxicity of polymeric micelles loaded with ADR[a]

Drug carrier system	Surviving cells (%, mean±S.D.)	
	29°C	37°C
Free ADR	85.4±2.5	63.0±11.4
PIPAAm–PBMA blank micelle	97.9±0.6	100.0±7.3
PIPAAm–PBMA micelle loaded with ADR	97.4±3.1	33.5±5.1
PIPAAm–PSt blank micelle	100.0±6.2	100.0±8.4
PIPAAm–PSt micelle loaded with ADR	87.5±5.2	97.9±3.1
Without carriers and drug	100.0±5.0	100.0±7.7

[a] ADR concentration=0.1 μg/ml, incubation time with drug=4 days.

Enhanced, active micelles and cell drug uptake by c tive routes. PIPA changes from hy core structural c upon heating abc shell collapse o not followed by structures, as m the observed ver ADR micelles u

Therefore, the comprising block ed hydrophobic thermally specifi enhancement elic drug release ini chemical propert to polymeric m separated microc shells and hydro main was indep for each feasible as a thermo-respc tions with targe heating. The core to structural dist inner core chemi expressed by rel tween outer shell tion of this coop to control targete initiated drug rel targeting cells, c tissue conditions tation manipulati

4. Conclusion

Two thermo-re prising PIPAAm c PBMA or PSt w PBMA and PIPA in organic solve demonstrated re dispersion meas

Enhanced, active interaction between the polymeric micelles and cells above the LCST may provide high drug uptake by cells through alternative, more effective routes. PIPAAm–PBMA micelles undergo both changes from hydrophilic to hydrophobic as well as core structural deformation, initiating drug release upon heating above the LCST. By contrast, the outer shell collapse of PIPAAm–PSt/ADR micelles was not followed by drug release, due to stable core structures, as mentioned above. This could explain the observed very low cytotoxicity of PIPAAm–PSt/ADR micelles under these conditions.

Therefore, thermo-responsive polymeric micelles comprising block copolymers of PIPAAm and selected hydrophobic segments are thought to express thermally specific drug action due to either uptake enhancement elicited by hydrophobic outer shells or drug release initiation regulated by the physical/chemical properties of inner cores. This is attributed to polymeric micellar structures comprising two separated microdomains, namely, hydrophilic outer shells and hydrophobic inner cores. Each microdomain was independently designed and synthesized for each feasible function. The outer shell functions as a thermo-responsive switch for controlling interactions with target cells upon local and transient heating. The core controls ON/OFF drug release due to structural distortion achieved by selection of the inner core chemistries. Ultimate drug bioactivity is expressed by release mediated by cooperation between outer shell and inner core behaviors. Regulation of this cooperation will extend the possibilities to control targeted drug delivery through selectively initiated drug release and enhanced uptake into the targeting cells, combined with local hyperthermic tissue conditions that, through modern instrumentation manipulations, cycle through the LCST.

4. Conclusion

Two thermo-responsive polymeric micelles comprising PIPAAm outer shells and inner cores of either PBMA or PSt were formed by dialyzing PIPAAm–PBMA and PIPAAm–PSt block copolymer solutions in organic solvents against water. These micelles demonstrated reversible intermicellar aggregation/dispersion measured by DLS and transmittance, responding to heating/cooling thermal cycles through the polymer outer shell LCST (32.5°C). PIPAAm–PBMA micelles with a relatively flexible inner core (lower T_g (20°C) of PBMA segments) compared to the outer shell LCST exhibited an abrupt increase in micropolarity and an abrupt decrease in microrigidity of the inner core upon heating above the LCST. In contrast, PIPAAm–PSt micelles with a glassy, rigid inner core (higher T_g (100°C) of PSt segments) compared to the outer shell LCST maintained constant values of lower micropolarity and higher microrigidity than those of PIPAAm–PBMA micelles over the entire temperature range. Both PIPAAm–PBMA micelles and PIPAAm–PSt micelles with the loaded hydrophobic drug, ADR in their inner cores were stable below the LCST. PIPAAm–PBMA/ADR micelle selectively released ADR upon heating above the LCST, while PIPAAm–PSt micelles did not. PIPAAm–PBMA/ADR micelles expressed selectively high in vitro cytotoxicity only when heated through the LCST, while PIPAAm–PSt/ADR micelles showed very low in vitro cytotoxicity, irrespective of a temperature change through the LCST.

From these results, correlating the T_g of the micellar hydrophobic segment comprising the inner core of thermo-responsive polymeric micelles with the outer shell polymer LCST is proposed to be an important means to control drug release with temperature changes. Further feasibility studies of thermo-responsive polymeric micelle controlled release are expected to demonstrate multiple functions for this targeting system, including ON/OFF stimuli-responsive behavior in combination with hyperthermic therapy.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (No. 10145104) on Priority Areas (No. 296, Bio-molecular Design for Biotargeting) from The Ministry of Education, Science, Sports and Culture. We would like to thank Dr. Takao Aoyagi for helpful discussion about polymer synthesis, and Prof. D.W. Grainger (Colorado State University) for critical suggestions regarding the manuscript.

References

[1] M. Heskins, J.E. Guillet, Solution properties of poly(*N*-isopropylacrylamide), J. Macromol. Sci. Chem. A2 (1968) 1441–1455.

[2] R. Yoshida, K. Uchida, Y. Kaneko, K. Sakai, A. Kikuchi, Y. Sakurai, T. Okano, Comb-type grafted hydrogels with rapid de-swelling response to temperature changes, Nature 374 (1995) 240–242.

[3] A.S. Hoffman, A.A. Afrassiabi, L.-C. Dong, Thermally reversible hydrogels: II. Delivery and selective release of substances from aqueous solution, J. Control. Release 4 (1986) 213–222.

[4] Y.H. Bae, T. Okano, S.W. Kim, A new thermo-sensitive hydrogel: Interpenetrating polymer networks from *N*-acryloylpyrrolidine and poly(oxyethylene), Markromol. Chem., Rapid Commun. 9 (1988) 185–189.

[5] H. Kanazawa, K. Yamamoto, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai, T. Okano, Temperature responsive chromatography using poly(*N*-isopropylacrylamide)-modified silica, Anal. Chem. 68 (1996) 100–105.

[6] H. Kanazawa, Y. Kashiwase, K. Yamamoto, Y. Matsushima, A. Kikuchi, Y. Sakurai, T. Okano, Temperature responsive liquid chromatography 2. Effect of hydrophobic groups in *N*-isopropylacrylamide copolymer-modified silica, Anal. Chem. 69 (1997) 823–830.

[7] T. Yakushiji, K. Sakai, A. Kikuchi, T. Aoyagi, Y. Sakurai, T. Okano, Graft architectural effects on thermo-responsive wettability changes of poly(*N*-isopropylacrylamide)-modified surfaces, Langmuire 14 (1998) 4657–4662.

[8] N. Yamada, T. Okano, H. Sakai, F. Karikusa, Y. Sawasaki, Y. Sakurai, Thermo-responsive polymeric surfaces; Control of attachment and detachment of cultured cells, Makromol. Chem., Rapid Commun. 11 (1990) 571–576.

[9] T. Okano, N. Yamada, H. Sakai, Y. Sakurai, A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(*N*-isopropylacrylamide), J. Biomed. Mater. Res. 27 (1993) 1243–1251.

[10] T. Okano, N. Yamada, M. Okuhara, H. Sakai, Y. Sakurai, Mechanism of cell detachment from temperature-modulated, hydrophilic–hydrophobic polymer surfaces, Biomaterials 16 (1995) 297–303.

[11] H. Maeda, L.W. Seymour, Y. Miyamoto, Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo, Bioconjugate Chem. 3 (1992) 351–361.

[12] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.

[13] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, S. Inoue, Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)–poly(aspartic acid) block copolymer, Cancer Res. 50 (1990) 1693–1700.

[14] M. Yokoyama, T. Okano, Y. Sakurai, H. Ekimoto, C. Shibazaki, K. Kataoka, Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood, Cancer Res. 51 (1991) 3229–3236.

[15] J. Overgaard, Combined adriamycin and hyperthermia treatment of a murine mammary carcinoma in vivo, Cancer Res. 36 (1976) 3077–3081.

[16] M.H. Shin, A. Cahan, J. Fogh, J.G. Fortner, Sensitivity of xenografts of human pancreatic adenocarcinoma in nude mice to heat and heat combined with chemotherapy, Cancer Res. 43 (1983) 4014–4018.

[17] J.N. Weinstein, R.L. Magin, M.B. Yatvin, D.S. Zaharko, Liposomes and local hyperthermia: selective delivery of methotrexate to heated tumors, Science 204 (1979) 188–191.

[18] B. Khoobehi, G.A. Peyman, W.G. McTurnan, M.R. Niesman, R.L. Magin, Externally triggered release of dye and drugs from liposomes into the eye. An in vitro and in vivo study, Opthalmology 95 (1988) 950–955.

[19] J.E. Chung, M. Yokoyama, K. Suzuki, T. Aoyagi, Y. Sakurai, T. Okano, Reversibly thermo-responsive alkyl-terminated poly(*N*-isopropylacrylamide) core-shell micellar structures, Colloids Surfaces (B: Biointerfaces) 9 (1997) 37–48.

[20] J.E. Chung, M. Yokoyama, T. Aoyagi, Y. Sakurai, T. Okano, Effect of molecular architecture of hydrophobically modified poly(*N*-isopropylacrylamide) on the formation of thermo-responsive core-shell micellar drug carriers, J. Control. Release 53 (1997) 119–130.

[21] S. Cammas, K. Suzuki, Y. Sone, Y. Sakurai, K. Kataoka, T. Okano, Thermo-responsive polymer nanoparticles with a core-shell micelle structure as site-specific drug carriers, J. Control. Release 48 (1997) 157–164.

[22] F. Kohori, K. Sakai, T. Aoyagi, M. Yokoyama, Y. Sakurai, T. Okano, Preparation and characterization of thermally responsive block copolymer micelles comprising poly(*N*-isopropylacrylamide–*b*-*dl*-lactide), J. Control. Release 55 (1998) 87–98.

[23] J.E. Chung, M. Yokoyama, M. Yamato, T. Aoyagi, Y. Sakurai, T. Okano, Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(*N*-isopropylacrylamide) and poly(butylmethacrylate), J. Control. Release (1998), in press.

[24] F. Kohori, K. Sakai, T. Aoyagi, M. Yokoyama, M. Yamato, Y. Sakurai, T. Okano, Control of adriamycin cytotoxic activity using thermally responsive polymeric micelles composed of poly(*N*-isopropylacrylamide–*co*-*N*,*N*-dimethylacrylamide)–*b*-poly(*dl*-lactide), Colloids Surfaces: special issue (1999), in press.

[25] Y.G. Takei, T. Aoki, K. Sanui, N. Ogata, T. Okano, Y. Sakurai, Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparations, Bioconjugate Chem. 4 (1993) 341–346.

[26] T. Okano, M. Katayama, I. Shinohara, The influence of hydrophobic and hydrophilic domains on water wettability of 2-hydrooxyethyl methacrylate/styrene copolymers, J. Appl. Polym. Sci. 22 (1978) 367–377.

[27] Y. Kaneko, K. Sakai, A. Kikuchi, R. Yoshida, Y. Sakurai, T. Okano, Influence of freely mobile grafted chain length on dynamic properties of comb-type grafted poly (*N*-isopropylacrylamic 7717–7723.

[28] R. Yoshida, K. phase transitio isopropylacryla Polym. Educ. 6

[29] L.M. Almeida Madeira, Fluid investigated wi cimer probe, B

[30] K.A. Zacharias Investigation o intramolecular (1982) 323–33

[31] R.L. Melnick, I S. Weinstein, tramolecular ex and biological

[32] M. Yamato, M Sakurai, T. Ok reorganization culture surface mer, J. Biomec

propylacrylamide) hydrogels, Macromolecules 28 (1995) 7717–7723.
[28] R. Yoshida, K. Sakai, T. Okano, Y. Sakurai, Modulating the phase transition temperature and thermosensitivity in *N*-isopropylacrylamide copolymer gels, J. Biomater. Sci. Polym. Educ. 6 (1994) 585–598.
[29] L.M. Almeida, W.L.C. Vaz, K.A. Zachariasse, V.M.C. Madeira, Fluidity of sarcoplasmic reticulum membranes investigated with dipyrenylpropane, an intramolecular excimer probe, Biochemistry 21 (1982) 5972–5977.
[30] K.A. Zachariasse, W.L.C. Vaz, C. Sotomayor, W. Kühnle, Investigation of human erythrocyte ghost membrane with intramolecular excimer probes, Biochem. Biophys. Acta 688 (1982) 323–332.
[31] R.L. Melnick, H.C. Haspel, M. Goldenberg, M. Greenbaum, S. Weinstein, Use of fluorescent probes that from intramolecular excimers to monitor structural changes in model and biological membranes, Biophys. J. 34 (1981) 499–515.
[32] M. Yamato, M. Okuhara, F. Karikusa, A. Kikuchi, Y. Sakurai, T. Okano, Signal transduction and cytoskelectal reorganization are required for cell detachment from cell culture surfaces grafted with a temperature-responsive polymer, J. Biomed. Mater. Res. 44 (1) (1999) 44–52.
[33] M.C. Alley, D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, M.R. Boyd, Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay, Cancer Res. 48 (1988) 589–601.
[34] L.-C. Dong, A.S. Hoffman, A novel approach for preparation of pH-sensitive hydrogels for enteric drug delivery, J. Control. Release 15 (1991) 141–152.
[35] G. Chen, A.S. Hoffman, Grafted copolymers that exhibit temperature-induced phased transitions over a wide range of pH, Nature 373 (1995) 49–52.
[36] L.D. Taylor, L.D. Cerankowski, Preparation of films exhibiting a balanced temperature dependence to permeation by aqueous solutions, J. Polym. Sci. Polym. Chem. 13 (1975) 2551–2570.
[37] D.C. Dong, M.A. Winnik, The Py scale of solvent polarities, Can. J. Chem. 62 (1984) 2560–2565.
[38] K. Kalyanasundaram, J.K. Thomas, Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar system, J. Am. Chem. Soc. 99 (1977) 2039–2044.